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<p><b>(54) Title: COMPOSITIONS THAT MEDIATE KILLING OF HIV-INFECTED CELLS</b></p> <p><b>(57) Abstract</b></p> <p>A method for directing a cytotoxic T cell to an HIV-1-infected cell, which comprises contacting the infected cell with a bispecific proteinaceous molecule comprising two binding domains, wherein the first binding domain comprises a CD4 domain or domains and the second binding domain comprises an anti-CD3 binding region.</p>			

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## COMPOSITIONS THAT MEDIATE KILLING OF HIV-INFECTED CELLS

### 5 INTRODUCTION

#### Technical Field

10 This application relates to pharmaceutical compositions useful in the treatment of human immunodeficiency virus (HIV) infections and particularly those useful for stimulating killing of HIV-infected cells by cytotoxic T lymphocytes.

#### Background

15 The primary immunologic abnormality resulting from infection by HIV is the progressive depletion and functional impairment of T lymphocytes expressing the CD4 cell surface glycoprotein (H. Lane *et al.*, Ann. Rev. Immunol. 3:477 [1985]). CD4 is a non-polymorphic glycoprotein with homology to the immunoglobulin gene superfamily (P. Maddon *et al.*, Cell 42:93 [1985]). Together with the CD8 surface antigen, CD4 defines two distinct subsets of 20 mature peripheral T cells (E. Reinherz *et al.*, Cell 19:821 [1980]), which are distinguished by their ability to interact with nominal antigen targets in the context of class I and class II major histocompatibility complex (MHC) antigens, respectively (S. Swain, Proc. Natl. Acad. Sci. 78:7101 [1981]; E. Engleman *et al.*, J. Immunol. 127:2124 [1981]; H. Spitz *et al.*, J. Immunol. 129:1563 [1982]; 25 W. Biddison *et al.*, J. Exp. Med. 156:1065 [1982]; and D. Wilde *et al.*, J. Immunol. 131:2178 [1983]). For the most part, CD4 T cells display the helper/inducer T cell phenotype (E. Reinherz, *supra*), although CD4 T cells characterized as cytotoxic/suppressor T cells have also been identified (Y. Thomas *et al.*, J. Exp. Med. 154:459 [1981]; S. Mauer *et al.*, Proc. Natl. Acad. Sci. USA 30 79:4395 [1982]; and A. Krensky *et al.*, Proc. Natl. Acad. Sci. USA 79:2365 [1982]). The loss of CD4 helper/inducer T cell function probably underlies the

profound defects in cellular and humoral immunity leading to the opportunistic infections and malignancies characteristic of the acquired immunodeficiency syndrome (AIDS) (H. Lane *supra*).

Studies of HIV-I infection of fractionated CD4 and CD8 T cells from normal donors and AIDS patients have revealed that depletion of CD4 T cells results from the ability of HIV-I to selectively infect, replicate in, and ultimately destroy this T lymphocyte subset (D. Klatzmann *et al.*, *Science* 225:59 [1984]). The possibility that CD4 itself is an essential component of the cellular receptor for HIV-I was first indicated by the observation that monoclonal antibodies directed against CD4 block HIV-I infection and syncytia induction (A. Dalgleish *et al.*, *Nature [London]* 312:767 [1984]; J. McDougal *et al.*, *Science* 231:382 [1986]; and the finding that HIV-I tropism can be conferred upon ordinarily non-permissive human cells following the stable expression of CD4 cDNA (P. Maddon *et al.*, *Cell* 47:333[1986]). Furthermore, the neurotropic properties of HIV-I, reflected by a high incidence of central nervous system dysfunction in HIV-I infected individuals (W. Snider *et al.*, *Ann. Neurol.* 14:403 [1983]), and the ability to detect HIV-I in the brain tissue and cerebrospinal fluid of AIDS patients (G. Shaw *et al.*, *Science* 227:177 [1985]; L. Epstein, *AIDS Res.* 1:447 [1985]; S. Koenig, *Science* 233:1089 [1986]; D. Ho *et al.*, *N. Engl. J. Med.* 313:1498 [1985]; J. Levy *et al.*, *Lancet* II:586 [1985]), appears to have its explanation in the expression of CD4 in cells of neuronal, glial and monocyte/macrophage origin (P. Maddon, *Cell* 47:444 [1986]; I. Funke *et al.*, *J. Exp. Med.* 165:1230 [1986]; B. Tourville *et al.*, *Science* 234:610 [1986]).

In addition to determining the susceptibility to HIV-I infection, the manifestation of cytopathic effects in the infected host cell appears to involve CD4. Antibody against CD4 was found to inhibit the fusion of uninfected CD4 T cells with HIV-I infected cells *in vitro*; moreover, the giant multinucleated cells produced by this event die shortly after being formed resulting in the depletion of the population of CD4 cells (J. Lifson *et al.*, *Science* 232:1123 [1986]). Formation of syncytia also requires gp120 expression, and can be elicited by co-culturing CD4-positive cell lines with cell lines expressing the HIV-I *env* gene in the absence of other viral structural or regulatory proteins (J. Sodroski *et al.*,

Nature 322:470 [1986]; J. Lifson *et al.*, Nature 323:725 [1986]). Thus, in mediating both the initial infection by HIV-I as well as eventual cell death, the interaction between gp120 and CD4 constitutes one of several critical entry points in the viral life cycle amenable to therapeutic intervention (H. Mitauya *et al.*, Nature 325:773 [1987]).

The known sequence of the CD4 precursor predicts a hydrophobic signal peptide, an extracellular region of approximately 370 amino acids, a highly hydrophobic stretch with significant identity to the membrane-spanning domain of the class II MHC beta chain, and a highly charged intracellular sequence of 40 residues (P. Madden, Cell 42:93 [1985]). The extracellular domain of CD4 consists of four contiguous regions each having amino acid and structural similarity to the variable and joining (V-J) domains of immunoglobulin light chains as well as related regions in other members of the immunoglobulin gene superfamily. These structurally similar regions of CD4 are termed the V<sub>1</sub>, V<sub>2</sub>, V<sub>3</sub> and V<sub>4</sub> domains (denominated 1-4 in Figure 1).

Some work preliminary to the present invention was described in a publication from the laboratory of the present inventors, namely J. Berg *et al.*, Proc. Natl. Acad. Sci. USA, 88:4723-4727. However, this publication describes only bispecific antibodies with complete Fc and CD4 domains and not other bispecific molecules. As noted in the publication, only clinical testing can determine whether such compounds that contain both CD4 and anti-CD3 regions along with other binding regions, such as those for the Fc receptor and MHC class II antigens, will be of clinical value.

Accordingly, it is an object of this invention to produce pharmaceutical compositions containing CD4 derivatives useful in the treatment of AIDS and related conditions, in a manner essentially unaffected by the extreme degree of genetic variation observed among various HIV-I isolates and their respective env polypeptides (J. Coffin, Cell 46:1 [1986]). In particular, it is an objective to prepare compositions containing molecules for directing cytotoxic T cells to cells exhibiting HIV antigens, e.g. HIV gp120, on their surfaces so that the infected cells can be destroyed using molecules that are particularly designed for clinical use.

SUMMARY OF THE INVENTION

The objects of this invention have been accomplished by providing pharmaceutical compositions containing a pharmaceutically acceptable carrier and an effective amount of a molecule that is bispecific and that can easily be produced by techniques of genetic engineering from readily available genetic sources. One specific binding region of the molecule, generally referred to herein as a binding domain, consists of a portion (or all) of a CD4 variable region that is capable of binding to HIV gp120 while the other binding region (binding domain) exhibits specific binding affinity for a cytotoxic T cell, typically via an anti-CD3 binding region of an antibody. The bispecific molecules used in compositions of the invention, referred to herein as "bispecs," are able to mediate the killing of cells infected with HIV virus of any strain by cytotoxic T cells of any specificity. It is no longer necessary to direct an antibody specifically to the HIV virus or to any particular class of T lymphocytes.

Several types of Bispecs are particularly preferred. These include single polypeptide chains with a CD4 region at one end and an anti-CD3 region at the other end, typically prepared by splicing CD4 variable region domains of a CD4 molecule onto anti-CD3 variable region domains of an anti-CD3 antibody, using techniques of genetic engineering. Alternatively, modified antibody molecules can be prepared, in which one arm of the antibody is derived from an anti-CD3 antibody and the other arm is derived from a CD4 molecule. These antibody molecules are preferably designed to be specifically useful in clinical situations by incorporating deletions of non-specific binding regions in, for example, the Fc region of the antibody chain, so that undesired binding, and thus indiscriminate cell killing, does not occur. Bispecs of the invention, as described in detail below, have been demonstrated to mediate killing of HIV infected cells in the presence of completely unrelated cytotoxic T lymphocytes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the structure of a CD4 molecule and of a typical immunoglobulin chain exemplified by the  $\gamma 1$  antibody chain. CD4 and  $\gamma 1$  are shown in the first two lines of the Figure 1. The third

line of Figure 1 shows the four immunoglobulin-like domains of CD4 as a separate soluble entity, as they can be prepared by techniques of recombinant engineering (soluble rCD4). The last two lines of Figure 1 show two fusion proteins formed by linking various CD4 domains to the constant region of the  $\gamma 1$  immunoglobulin chain. The first of these two representations, indicated by  $CD4_{2\gamma 1}$ , consists of the first two domains of CD4 linked to the constant region of  $\gamma 1$ . The last line of the Figure, identified as  $CD4_{4\gamma 1}$ , shows a construct in which the first four domains of have been attached to the constant region of  $\gamma 1$ .

Figure 2 is a schematic representation of a bispecific antibody. The left arm of the molecule consists of the fusion protein  $CD4\gamma 1$  linked to  $\kappa$  light chain by a disulfide bridge; the right arm consists of a  $\gamma 1, \kappa$  pair. The left arm binds to HIV gp120 of any strain. The right arm of the bispecific antibody binds to CD3, a component of the T cell receptor, and, thereby, activates the cytotoxic T cell. CD4.1, CD4.2, CD4.3, and CD4.4 are immunoglobulin-like domains of CD4; CH1, CH2, and CH3 are constant region domains of  $\gamma 1$  chains; VH is the variable region of  $\gamma 1$  heavy chain; VL is the variable region of  $\kappa$ .

Figure 3 is a series of schematic representations of autoradiographs.  
(a) Autoradiograph obtained after SDS-polyacrylamide gel electrophoresis of [<sup>35</sup>S]methionine-labeled immunoglobulin chains produced by hybridoma M-T301 (lane 2) and the transfectoma (lane 3). Lane 1: molecular weight standards. Cells were incubated for 20 min in RPMI containing [<sup>35</sup>S]methionine. Proteins were immunoprecipitated from lysed cells using goat antibody to mouse immunoglobulin followed by S. aureus. The precipitate was reduced and analyzed on a 10% gel. The band at a mol. weight of circa 40,000 presumably represents a truncated immunoglobulin chain encoded by a silent allele; it is not secreted [see (b)]. (b) Fluorograph obtained after SDS-polyacrylamide gel electrophoresis of [<sup>35</sup>S]methionine-labeled immunoglobulin from the supernatants of hybridoma M-T301 (lane 1) and the transfectoma (lane 2). Material in lanes 1 and 2 was reduced before being loaded onto the gel. Far left lane: molecular weight standards. (c) Non-reduced precipitates from M-T301 (Lane 3) and the transfectoma (lane 4). Positions 1, 2, and 3 indicate M-T301 immunoglobulin, bispecific antibody, and  $CD4\gamma 1, \kappa$  tetramers, respectively. (d) Lane 4 of Figure

3(c) was cut out and rerun under reducing conditions. (e) Transfectoma supernatant purified over an anti-CD4 column, electrophoresed non-reduced or after reduction. (f) Non-reduced material electrophoresed as in Figure 3(e) rerun under reducing conditions. Positions 1 and 2 indicate bispecific antibody and 5 CD4 $\gamma$ 1,κ dimers, respectively. Staining in 2(e) and 2(f) was with Commassie blue.

#### DESCRIPTION OF SPECIFIC EMBODIMENTS

10 Although AIDS patients lose HIV-specific cytotoxic T cells, their remaining CD8-positive T lymphocytes maintain cytotoxic function. To exploit this fact, we have constructed bispecific molecules, usually a modified form of antibodies, that direct cytotoxic T lymphocytes of any specificity to cells that express gp120. These bispecific molecules ("bispecs") comprise an anti-T-cell-specific-antigen binding region and a CD4 region; e.g., one heavy/light chain pair from an antibody to a CD3 antigen linked to a heavy chain whose variable region has been replaced with sequences from CD4 plus a second light chain. CD3 is 15 part of the antigen receptor on T cells and is responsible for signal transduction and is used throughout this specification as the exemplary (and preferred) 20 cytotoxic-T-cell-specific antigen. In the presence of bispecific molecules of these specificities, T cells of irrelevant specificity effectively lyse HIV-infected cells, as shown by the examples below in which this cytotoxicity has been demonstrated.

25 It was previously known that human peripheral blood lymphocytes can kill HIV-infected cells *in vitro* in the presence of monoclonal anti-gp120 antibodies chemically cross-linked to monoclonal antibodies to CD3. We have improved upon this scheme in two ways. First, we have replaced the chemical linkage with a biochemical one. By transfection we produced a cell line expressing molecules of both specificities. This line secretes, among other products, the desired bispecific molecules. Second, we have replaced the anti- 30 gp120 monoclonal antibody, which was strain-specific and, therefore, of limited therapeutic potential, by a CD4 derivative, such as a CD4 $\gamma$ 1 chain; in this example the heavy antibody chain consists of the first four immunoglobulin-like

d mains of CD4 fused to the whole constant region of mouse  $\gamma 1$  chain. HIV cannot mutate out f the binding capability of CD4 without losing its ability to infect cells via their membrane CD4 molecule, so that this and other bispecs will be effective against any HIV strain.

5 The word "domain" is used in this specification in the same manner as it is used in biochemistry to describe regions of molecules, usually proteins, that interact with other molecules or have other properties restricted to one (or more) region of the total molecule. In its broadest sense, a domain is simply a region of a molecule that is associated with a particular physical or chemical 10 property, such as binding to another molecule. This region is generally but not always comprised of a single continuous segment of the amino acid sequence that makes up the total protein. As used herein, a "domain" preferably refers to such a continuous segment. In the field of immunology, "domain" has come to have a more specialized meaning, namely one of the roughly spherical regions of an 15 antibody chain that are linked to each other to form an immunoglobulin chain (or a similar region of another member of the immunoglobulin superfamily of molecules, such as CD4). The particular meaning of domain as used herein will be clear from the context, but generally domain is used in the broader sense 20 defined above unless it is used to refer to one of the immunoglobulin (or immunoglobulin-like) regions of an antibody chain or CD4 molecule that is specifically referred to as a domain in the scientific literature. For exemplification 25 of the meaning of domain as it is used in context of immunoglobulin and CD4 segments in the scientific literature, see the publications discussed in the Background section of this specification.

25 In addition to bispecific antibodies, the invention can be practiced with other molecules that are bispecific in the same manner as the antibodies described above. For example, the immunoglobulin-like domains of a CD4 molecule can be linked to a molecule retaining its specificity for CD3 in any fashion, including using linkers (usually short polypeptide chains), to provide a 30 bispec of the invention. A particularly preferred bispec is prepared as a fusion protein in which one end of the molecule is a CD4 domain or domains and the other end of the molecule is a binding region for CD3. Such molecules can

readily be prepared as fused proteins by genetic engineering by linking coding regions together, such as the coding region for one or more domains of CD4 and the coding region for at least the binding region of one strand, preferably the heavy chain variable region, of an anti-CD3 antibody. Recent work has shown 5 that single-stranded antibody chains retain specificity for their specific antigen (discussed below in more detail). See, for example, the techniques described in Ward *et al.*, *Nature*, 341:544 (1989). Expression of the resulting polynucleotide results in production of a fused protein containing two regions with the desired primary amino acid structures.

10 For example, a single-chain polypeptide of the invention can be prepared by linking together the coding regions for at least one CD4 variable region domain, for  $V_H$  and  $V_L$ , and for  $C_\alpha$ . The resulting single polypeptide will thus contain both  $V_L$  and  $V_H$ . This is readily achieved by inserting a polypeptide linker between  $V_H$  and  $V_L$ . Examples of molecules prepared in this manner are given 15 below. A particularly preferred example, D- $V_H$ - $V_L$ - $C_\alpha$ , has been shown to be effective by colleagues of the present inventors. In these molecules,  $V_H$  and  $V_L$  are linked via a short peptide linker in order to provide sufficient flexibility for the antibody binding site to form. However, the CD4 domain can be attached directly 20 to the N terminus of the  $V_H$  domain. In the molecule that has been expressed, the CD4 domain has its leader sequence for secretion from mammalian cells, but the leader sequences of the  $V_H$  and  $V_L$  are not present. In preferred embodiments, the  $C_\alpha$  region is retained in order to assist in folding and stability of the antibody part 25 of the molecule, even if other deletions occur, as discussed in more detail below. Constructs that comprise entire antibody and CD4 domains (rather than parts of domains) are also preferred for ease of folding, expression, and genetic manipulation.

30 Whether any particular construct retains its ability after folding to carry out the desired binding functions can readily be determined by simple binding experiments. For example, detectably labeled gp120 molecules can be used in a variety of well-known techniques to determine the ability of the CD4 portion of the molecule to bind gp120. Similarly, detectably labeled CD3 antigen can be used to determine whether the anti-CD3 portion of the molecule retains its binding

ability. If desired for higher binding affinity or greater specificity, a genetic construct can be prepared that encodes two proteins, one being the fused CD4/antibody chain (e.g., with an antibody heavy chain) and the other being the remaining anti-CD3 antibody chain (e.g., the light chain). Expression of the two proteins concurrently will result in assembly of the light and heavy chains to form one light/heavy chain pair (i.e., one arm of an antibody against CD3) with the CD4 domain being attached to one of the antibody chains.

When assembled in this fashion, it is preferred to have the antibody binding domain at the N terminus of the fused molecule and (in some embodiments) to include at least part of the constant region of the two antibody chains for ease of post-expression assembly. However, other embodiments eliminate all or part of one or more constant region domains, as discussed in more detail below, in order to eliminate undesired binding of the antibody Fc region to other cells and/or molecules. The CD4 region is normally attached in such constructs at the C terminus, although the alternative order can occur as well. Such genetic constructs are prepared in the same manner as is described in detail below for the production of bispecific antibodies of the invention except for the order and/or selection of the segments that are spliced together.

Another preferred embodiment uses the concept described by Ward *et al.*, *Nature*, 341:544 (1989), in which single antibody chains are prepared that retain binding specificity, where the first or the first two domains of the heavy chain are linked by a peptide bridge to the first domain of or the complete light chain. The resulting single antibody chain can be readily fused to a CD4 domain or domains as described herein, using, in preferred embodiments, the same linking groups to attach the two antibody variable regions. The resulting single-chain protein, which is readily produced by genetic engineering and expression, has a structure such as D-V<sub>L</sub>-V<sub>H</sub>-C<sub>κ</sub>, D-V<sub>H</sub>-V<sub>L</sub>-C<sub>κ</sub>, D-V<sub>H</sub>-V<sub>L</sub>-C<sub>λ</sub>, V<sub>H</sub>-V<sub>L</sub>-C<sub>κ</sub>-D, V<sub>L</sub>-V<sub>H</sub>-C<sub>κ</sub>-D, V<sub>H</sub>-V<sub>L</sub>-C<sub>λ</sub>-D, and other similar combinations in which a portion (D) of CD4 that retains its binding affinity for gp120 is fused to variable (V; heavy and light chains) and constant (C) domains of an anti-CD3 antibody. Preferred fusions have a CD4 domain or domains at one terminus of the polypeptide chain and the antibody domains at the other terminus. Additional amino acids can be

present in central portions of the polypeptide (i.e., between CD4 and antibody domains) without adversely affecting binding affinities.

Bispecs of the invention can readily be prepared using existing sources of genetic material encoding the indicated specific molecules. However, 5 the invention also encompasses variations derived from the original material that has been modified by techniques of genetic and protein engineering to provide additional desirable properties in the resulting bispecs. Those bispecs that have the same amino acid sequence (in their relevant portions) as can be found in naturally occurring CD4 and anti-CD3 molecules are referred to herein as 10 "natural-sequence bispecs." This invention, however, is also concerned with amino acid sequence variants of natural-sequence bispecs. Amino acid sequence variants of bispecs are prepared with various objectives in mind, including increasing the affinity of the bispec for its binding partner, facilitating the stability, purification and preparation of the bispec, increasing its plasma half life, 15 improving therapeutic efficacy as described above in the background, introducing additional functionalities and lessening the severity or occurrence of side effects during therapeutic use of the bispec. Amino acid sequence variants of bispecs fall into one or a combination of insertional, substitutional, and deletional variants, as will be discussed in detail below.

20 In a specific embodiment of a bispec of the invention, one or more CD4 immunoglobulin-like domains, which may be homologous either to the constant or to the variable region domains, or any other fragment of CD4 (as long as the CD4 sequence retains its ability to bind gp120) is conjugated with an immunoglobulin constant region sequence. Immunoglobulins and certain variants 25 thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner *et al.*, *Nature* 298:286 (1982); EP 120,694; EP 125,023; Morrison, *J. Immun.* 123:793 (1979); Kohler *et* *al.*, *P.N.A.S. USA* 77:2197 (1980); Raso *et al.*, *Cancer Res.* 41:2073 (1981); Morrison *et al.*, *Ann. Rev. Immunol.* 2:239 (1984); Morrison, *Science* 229:1202 (1985); Morrison *et al.*, *P.N.A.S. USA* 81:6851 (1984); EP 255,694; EP 30 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. Patent 4,444,878; WO 88/03565; and EP 68,763 and

5 references cited therein. For production of single antibody chains that retain binding specificity, where the first or the first two domains of the heavy chain are linked by a peptide bridge to the first domain of or the complete light chain, see Ward *et al.*, *Nature*, 341:544 (1989). All of these patents and other publications are herein incorporated by reference.

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Ordinarily, the domains of CD4 that are homologous to immunoglobulins and extracellular in their native environment are fused C-terminally to the N-terminus of the constant region of immunoglobulins in place of the variable region(s) thereof, retaining at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Immunoglobulins and other polypeptides having enhanced plasma half life are fused to the extracellular or ligand binding domains of other CD4 variants in the same fashion.

15

15 The boundary domains for the CD4 V-like regions (V1-V4) are, respectively, about 100-109, about 175-184, about 289-298, and about 360-369 (based on the precursor CD4 amino acid sequence in which the initiating met is -25). For a full disclosure of the CD4 sequence, which is well known to those familiar with HIV infections and the CD4 molecule, see Figures 1 and 2 of PCT application US 88/03414 published under the International Publication No. WO 89/02922 on 6 April 1989. This PCT publication, which is herein incorporated by reference, describes the preparation of immunoglobulin chains containing various CD4 domains and the constant region of an antibody, but is not directed to combinations specifically involving anti-CD3 binding regions or to the targeting of cytotoxic T cells to HIV-infected cells.

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CD4 sequences containing any of the CD4 domains involved in binding to gp120 are fused to the immunoglobulin sequence. It is preferable that V1V2 or V1V2V3V4 be fused at its C-terminus to the immunoglobulin constant region. The precise site at which the fusion is made is not critical; the boundary domains noted herein are for guidance only and other sites neighboring or within the V regions may be selected in order to optimize the secretion or binding characteristics of the CD4. The optimal site will be determined by routine

experimentation. Unless the first constant region domain of the immunoglobulin heavy chain is deleted, or a light chain is provided, the fusions are generally expressed intracellularly, but a great deal of variation is encountered in the degree of secretion of the fusions from recombinant hosts. For instance, the following table (Table 1) shows various CD4 immunoglobulin fusions that were obtained in PCT/US88/03414. These immunoglobulin chains were referred as "immuno-adhesons," and this terminology is retained here to refer to single antibody chains consisting of one or more CD4 domains linked to the constant region of an immunoglobulin chain. In all of these examples of CD4 immuno-adhesons, the CD4 signal was used to direct secretion from 293 cells. Lower case m represent murine origin, while the lower case h designates human origin. V and C are abbreviations for immunoglobulin variable and constant domains respectively. The numerical subscripts indicate the number of parenthetical units found in the designated multimer. It will be understood that the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins. The CD4 immuno-adhesons typically contained either the first N-terminal 366 residues of CD4 (CD4<sub>4</sub>) or the first 180 N-terminal residues of CD4 (CD4<sub>2</sub>) linked at their C-terminus to the  $\kappa$  (light) chain or IgG1 heavy chain constant region ( $\gamma$ 1).

20

Table I

<u>Transfected Gene</u>	<u>Secreted Product</u>
$\text{aV}_\kappa\text{C}_\kappa$	$\text{aV}_\kappa\text{C}_\kappa$ and/or $(\text{aV}_\kappa\text{C}_\kappa)_2$
$\text{aV}_\gamma\text{C}_\gamma$	ND
$\text{aV}_\kappa\text{C}_\kappa + \text{aV}_\gamma\text{C}_\gamma$	$(\text{aV}_\kappa\text{C}_\kappa)_2(\text{aV}_\gamma\text{C}_\gamma)_2 +$ $\text{aV}_\kappa\text{C}_\kappa$ and/or $(\text{aV}_\kappa\text{C}_\kappa)_2$
$\text{hCD4}\text{-}\text{aC}_\kappa$	$\text{hCD4}\text{-}\text{aC}_\kappa$ and/or $(\text{hCD4}\text{-}\text{aC}_\kappa)_2$
$\text{hCD4}\text{-}\text{aC}_\gamma$	ND
$\text{hCD4}\text{-}\text{aC}_\kappa + \text{hCD4}\text{-}\text{aC}_\gamma$	$(\text{hCD4}\text{-}\text{aC}_\kappa)_2(\text{hCD4}\text{-}\text{aC}_\gamma)_2 +$ $\text{hCD4}\text{-}\text{aC}_\kappa$ and/or $(\text{hCD4}\text{-}\text{aC}_\kappa)_2$

	hCD4-hC <sub>n</sub>	hCD4-hC <sub>n</sub> and/or (hCD4-hC <sub>n</sub> ) <sub>2</sub>
	hCD4-hC <sub>γ1</sub>	(hCD4-hC <sub>γ1</sub> ) <sub>2</sub>
5	hCD4-hC <sub>n</sub> + hCD4-hC <sub>γ1</sub>	(hCD4-hC <sub>n</sub> ) <sub>2</sub> (hCD4-hC <sub>γ1</sub> ) <sub>2</sub> + hCD4-hC <sub>n</sub> and/or (hCD4-hC <sub>n</sub> ) <sub>2</sub>
	αV <sub>n</sub> C <sub>n</sub> + hCD4-hC <sub>γ1</sub>	(αV <sub>n</sub> C <sub>n</sub> ) <sub>2</sub> (hCD4-hC <sub>γ1</sub> ) <sub>2</sub> + αV <sub>n</sub> C <sub>n</sub> and/or (αV <sub>n</sub> C <sub>n</sub> ) <sub>2</sub>
	<u>*ND = Not detected</u>	

It can be seen that the CD4/human-heavy-chain immunoadheson was secreted as a dimer whereas the analogous murine construction was not detected (this does not exclude the intracellular accumulation of the protein, however). These CD4-IgG immunoadheson chimeras are readily secreted wherein the CD4 epitope is present fused to one or more light or heavy chains, including heterotetramers wherein up to and including all four variable region analogues are derived from CD4. Where a separate light-heavy chain non-CD4 variable domain is also present, a heterofunctional antibody thus is provided. In the present invention, the heterofunctional antibody is a bispec when the separate heavy-light chain is obtained from an anti-CD3 antibody. Such antibodies are available commercially, as are hybridomas that contain genetic information encoding anti-CD3 antibodies. Additionally, production of new anti-CD3-producing cell lines can readily be accomplished by routine immunological techniques.

Various exemplary hetero- and chimeric bispecific antibodies produced in accordance with this invention (as well as single polypeptide chains used to prepare bispecs) are schematically diagrammed below. "D" means at least a portion of the extracellular domain of CD4 containing its ligand binding site; V<sub>L</sub>, V<sub>H</sub>, C<sub>L</sub> and C<sub>H</sub> represent light or heavy chain variable or constant domains of an immunoglobulin; n is an integer; and Y designates a covalent cross-linking moiety.

- (a)  $DC_L$ ;
- (b)  $DC_L-DC_L$ ;
- (c)  $DC_H-[CD_H, DC_L-DC_H, DC_L-V_HC_H, V_LC_L-DC_H, \text{ or } V_LC_L-V_HC_H]$ ;
- (d)  $DC_L-DC_H-[DC_H, DC_L-DC_H, DC_L-V_HC_H, V_LC_L-DC_H, \text{ or } V_LC_L-$   
5  $V_HC_H]$ ;
- (e)  $DC_L-V_HC_H-[CD_H, DC_L-DC_H, DC_L-V_HC_H, V_LC_L-DC_H, \text{ or } V_LC_L-$   
 $V_HC_H]$ ;
- (f)  $V_LC_L-DC_H-[CD_H, DC_L-DC_H, DC_L-V_HC_H, V_LC_L-DC_H, \text{ OR } V_LC_L-$   
 $V_HC_H]$ ;
- 10 or
- (g)  $[D-Y]_n-[V_LC_L-V_HC_H]_2$ .

The structures in this list of formulas show only key features; e.g., they do not show joining ( $J$ ) or other domains of the immunoglobulins, nor are disulfide bonds shown. These are omitted in the interests of brevity. However, 15 where such domains are required for binding activity they shall be construed as being present in the ordinary locations which they occupy in the CD4, bispec, or immunoglobulin molecules as the case may be. These examples are representative of divalent antibodies; more complex structures would result by employing immunoglobulin heavy chain sequences from other classes, e.g., IgM. The immunoglobulin  $V_LV_H$  antibody combining site, also designated as the companion immunoglobulin, preferably is capable of binding to a predetermined antigen. Suitable companion immunoglobulin combining sites and fusion partners are obtained from IgG-1, -2, -3, or -4 subtypes, IgA, IgE, IgD or IgM.

20 A preferred embodiment of a fused CD4/immunoglobulin chain for use in preparing a bispec of the invention is a fusion of an N-terminal portion of CD4, which contains the binding site for the gp120 envelope protein of HIV, to the C-terminal  $F_c$  portion of an antibody, containing the effector functions of immunoglobulin  $G_1$ . There are two preferred embodiments of this sort with 25 respect to the portion of the antibody chain that is used; in one, the entire heavy chain constant region is fused to a portion of CD4; in another, a sequence beginning in the hinge region just upstream of the papain cleavage site which

defines IgG F<sub>c</sub> chemically (residue 216, taking the first residue of heavy chain constant region to be 114 [Kabat *et al.*, "Sequences of Proteins of Immunological Interest" 4th Ed., 1987], or analogous sites of other immunoglobulins) is fused to a portion of CD4. These embodiments are described in the examples.

5 More particularly, those variants in which one or more immunoglobulin-like domains of CD4 are substituted for the variable region of an immunoglobulin chain are useful in preparing bispecs of the invention. These chimeras are constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; Munro, *Nature* 312: (13 December 1984); Neuberger *et al.*, *Nature* 312: (13 December 1984); Sharon *et al.*, *Nature* 309: (24 May 1984); Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Morrison *et al.*, *Science* 229:1202-1207 (1985); and Boulianne *et al.*, *Nature* 312:643-646 (13 December 1984). The DNA encoding the CD4 immunoglobulin-like domain(s) is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the mature CD4 polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for CD4 (where the native CD4 signal is employed).  
10 This DNA fragment then is readily inserted into DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, tailored by deletional mutagenesis. Preferably, this is a human immunoglobulin when the variant is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily  
15 available from cDNA libraries or is synthesized. See for example, Adams *et al.*, *Biochemistry* 19:2711-2719 (1980); Gough *et al.*, *Biochemistry* 19:2702-2710 (1980); Dolby *et al.*, *P.N.A.S. USA* 77:6027-6031 (1980); Rice *et al.*, *P.N.A.S. USA* 79:7862-7865 (1982); Falkner *et al.*, *Nature* 298:286-288 (1982); and Morrison *et al.*, *Ann. Rev. Immunol.* 2:239-256 (1984). All publications cited in  
20 this paragraph are herein incorporated by reference.  
25  
30

DNA encoding the immunoglobulin or immunoadheson chimeric chain(s) is transfected into a host cell for expression. If the host cell is producing

an immunoglobulin prior to transfection, then one need only transfect with the CD4 domains fused to light or to heavy chain to produce a heteroantibody. The aforementioned immunoglobulins having one or more arms bearing the CD4 domain and one or more arms bearing companion variable regions specific for CD3 result 5 in dual specificity for gp120 of any source and for a CD3 antigen. These are produced by the above-described recombinant methods or by in vitro procedures. In the latter case, for example,  $F(ab')_2$  fragments of the adheson fusion and an immunoglobulin are prepared, the  $F(ab')_2$  fragments converted to Fab' fragments by reduction under mild reducing conditions, and then reoxidized in each other's 10 presence under acidic conditions in accord with methods known *per se*. See also U.S. Patent 4,444,878, which is incorporated herein by reference.

Additionally, procedures are known for producing intact heteroantibodies from immunoglobulins having different specificities. These 15 procedures can be adopted for the in vitro production of bispecs by simply substituting the CD4-containing chains for one of the previously employed immunoglobulins.

In an alternative method for producing a heterofunctional antibody, host cells producing a CD4-immunoglobulin fusion, e.g., transfected myelomas, also are fused with B cells or hybridomas which secrete antibody having the 20 desired companion specificity for CD3 antigen. Heterobifunctional antibody is recovered from the culture medium of such hybridomas, and thus can be produced somewhat more conveniently than by conventional in vitro resorting methods (EP 68,763).

As was previously mentioned, the present invention contemplates 25 not only natural-sequence bispecs, but variants in which there have been single, multiple, or combinations of insertions, deletions, and/or substitutions of amino acids in the naturally derived sequences. Insertional amino acid sequence variants are those in which one or more amino acid residues extraneous to the bispec are introduced into a predetermined site in the bispec including the C or N terminus of 30 any polypeptide chain. Such variants, particularly when the insertions are lengthy, are referred to as fusions of the bispec and a different polypeptide. Such fusions

contain sequences other than those which are normally found in the bispec at the inserted position.

Another class of bispec variants comprises deletional variants. Deletions are characterized by the removal of one or more amino acid residues from a given sequence. Typically, the transmembrane and cytoplasmic domains of CD4 are deleted. When such deletions are desired in the case of CD4, at least residues 368 to 395 (the transmembrane region), and ordinarily 396-433 as well (the cytoplasmic domain), will be deleted.

Another preferred deletion is one which reduces or eliminates the ability of CD4 to bind to MHC class II antigens. The amino acid residues associated with binding are numbers 19, 89, and 165 of the CD4 molecule. Mutations of these residues abrogate MHC binding. CD4 deletions affecting MHC class II antigen binding with a CD4 molecule is described in Fleury *et al.*, Cell 66:1037.

Other deletions can be used with the present invention as long as the binding specificity or other desired properties, such as effector functions, are retained. A preferred class of compounds encompasses bispecs derived from antibodies in which the binding region for Fc receptor is deleted or modified to reduce undesired binding. Such deletions can be of one or more entire domains of the Fc region or can a deletion or mutation of part of a domain or even a single amino acid. For example, a preferred mutation would be to change residue 235 of IgG from leu to glu, as demonstrated and described in Alegre *et al.*, J. Immunol. 148:3461. When an entire domain is to be deleted, the CH2 domain, which is the domain that interacts the Fc receptor, should be deleted. Whether or not any given deletion results in a bispec with the desired binding properties can readily be tested using the procedures described in the following examples to determine whether the resulting molecule is capable of directing cytotoxic T cells to HIV-infected cells.

Substitutional variants are those in which at least one residue in a sequence has been removed and a different residue inserted in its place. For example, the native N-terminal residue for mature CD4 is now known to be lysine. Thus, the normal sequence shown in Figure 1 of PCT/US88/03414, with

an N-terminal asparagine, is an amino acid sequence variant of native mature CD4. Table 2 below describes substitutions which in general will result in fine modulation of the characteristics of the CD4 antigen or anti-CD3 binding region.

5

TABLE 2

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
	Ala	ser
	Arg	lys
	Asn	gln; his
	Asp	glu
10	Cys	ser; ala
	Gln	asn
	Glu	asp
	Gly	pro
	His	asn; gln
	Ile	leu; val
	Leu	ile; val
15	Lys	arg; gln; glu
	Met	leu; ile
	Phe	met; leu; tyr
	Ser	thr
	Thr	ser
	Trp	tyr
20	Tyr	trp; phe
	Val	ile; leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in adhesion properties will be those in which (a) a hydrophilic residue, e.g., seryl or threonine, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, pheylalanyl, valyl or alanyl; (b) a cysteinyl or prolyl is substituted for (or by) any

other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanyl, is substituted for (or by) one not having a side chain, e.g., glycyl.

5 It will be amply apparent from the foregoing discussion that substitutions, deletions, insertions or any combination thereof are easily introduced to arrive at a final construct. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the CD4 or anti-CD3 molecules, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. However, variant bispecs also are prepared 10 by in vitro synthesis. Obviously, variations made in the DNA encoding the variant bispecs must not place the sequence out of reading from and preferably will not create complementary regions that could produce secondary mRNA structure deleterious to expression (EP 75 444 A). The CD4-derived region or 15 regions of the variants typically exhibit the same gp120 binding activity as does the naturally occurring prototype, although variants also are selected in order to modify the characteristics of the CD4 adheson as indicated above. For example, the regions of the CD4 molecule that are responsible for binding to class II major histocompatibility antigen (MHC) can be deleted in order to eliminate binding to 20 undesired locations in vivo. Similar considerations exist for the anti-CD3 region or regions.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in 25 order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed adheson variants screened for the optimal combination of desired activities. Techniques for making substitution mutations are predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis.

The DNA encoding CD4 is obtained by known procedures. See 30 Williams, Immunol. Today 8:298-303 (1987) and citations therein. In general, prokaryotes are used for cloning of CD4 variant DNA sequences. For example, E. coli strain SR101 (for propagating m13 phage, a  $\lambda$ -resistant strain f JM 101;

Messing *et al.*, *Nucl. Acids. Res.* 9(2):309-321 [1981]); and *E. coli* K12 strain 294 (ATCC No. 31446) are particularly useful. Other microbial strains which may be used include *E. coli* B, UMI01 and *E. coli*  $\chi$ 1776 (ATCC No. 31537). These examples are illustrative rather than limiting.

5 DNA encoding the various constructs used in preparing polypeptides of the invention is inserted for expression into vectors containing promoters and control sequences which are derived from species compatible with the intended host cell. The vector will ordinarily, but need not, carry a replication site as well as one or more marker sequences which are capable of providing phenotypic 10 selection in transformed cells. For example, *E. coli* is typically transformed using a derivative of pBR322 which is a plasmid derived from an *E. coli* species (Bolivar *et al.*, *Gene* 2:95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed 15 cells. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA constructions.

Promoters suitable for use with prokaryotic hosts illustratively include the  $\beta$ -lactamase and lactose promoter systems (Chang *et al.*, *Nature* 275:615 [1978]; and Goeddel *et al.*, *Nature* 281:544 [1979]), alkaline phosphatase, 20 the tryptophan (trp) promoter systems (Goeddel, *Nucleic Acids Res.* 8:4057 [1980] and EPO Appln. Publ. No. 36,776) and hybrid promoters such as the tac promoter (H. de Boer *et al.*, *Proc. Natl. Acad. Sci. USA* 80:21-25 [1983]). However, other 25 functional bacterial promoters are suitable. Their nucleotide sequences are generally known, thereby enabling a skilled worker operably to ligate them to DNA encoding the adheson variant using linkers or adaptors to supply any required restriction sites (Siebenlist *et al.*, *Cell* 20:269 [1980]). Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antigen.

In addition to prokaryotes, eukaryotic microbes such as yeast 30 cultures also are useful as cloning or expression hosts. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in

Saccharomyces, the plasmid YRp7, for example, (Stinchcomb *et al.*, *Nature* 282:39 [1979]; Kingsman *et al.*, *Gene* 7:141 [1979]; Tschemper *et al.*, *Gene* 10:157 [1980]) is commonly used. This plasmid already contains the *trp1* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics* 85:12 [1977]). The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective means of selection by growth in the absence of tryptophan.

10 Suitable promoting sequences for use with yeast hosts include the  
promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.*  
255:2073 [1980]) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.*  
7:149 [1968]; and Holland, *Biochemistry* 17:4900 [1978]), such as enolase,  
glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase,  
phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase,  
pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and  
glucokinase.

20 Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Publication No. 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

25

Promoters for controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers et al., *Nature* 273:113 (1978). The immediate early promoter of the human

cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway, P.J. et al., Gene 18:355-360 (1982). Of course, promoters from the host cell or related species also are useful herein.

5 DNA transcription in higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are *cis* acting elements of DNA, usually from about 10 to 300bp, that act to increase the transcription initiation capability of a promoter. Enhancers are relatively orientation and position independent having been found 5' (Laimins, L. et al., Proc. Natl. Acad. Sci. 78:993 [1981]) and 3' (Lusky, M.L., et al., Mol. Cell Bio. 3:1108 [1983]) to the transcription unit, within an intron (Banerji, J.L., et al., Cell 33:729 [1983]) as well as within the coding sequence itself (Osborne, T.F., et al., Mol. Cell Bio. 4:1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 10 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side 15 of the replication origin, and adenovirus enhancers.

20 Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding the adhesor.

25 Expression vector systems generally will contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase or neomycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of 30 selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented medium. Two examples are: CHO DHFR cells and mouse LTK cells. These cells lack the ability to grow without the addition of such nutrients as

thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented medium. An alternative to supplementing the medium is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, Southern P. and Berg, P., *J. Molec. Appl. Genet.* 1:327 (1982), mycophenolic acid, Mulligan, R.C. and Berg, P., *Science* 209:1422 (1980) or hygromycin, Sugden, B. et al., *Mol. Cell. Bio.* 5:410-413 (1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively.

"Amplification" refers to the increase or replication of an isolated region within a cell's chromosomal DNA. Amplification is achieved using a selection agent e.g., methotrexate (MTX) which inactivates DHFR. Amplification for the making of successive copies of the DHFR gene results in greater amounts of DHFR being produced in the face of greater amounts of MTX. Amplification pressure is applied notwithstanding the presence of endogenous DHFR, by adding ever greater amounts of MTX to the media. Amplification of desired gene can be achieved by cotransfected a mammalian host cell with a plasmid having a DNA encoding a desired protein and the DHFR or amplification gene permitting cointegration. One ensures that the cell requires more DHFR, which requirement is met by replication of the selection gene, by selecting only for cells that can grow in the presence of ever-greater MTX concentration. so long as the gene encoding a desired heterologous protein has cointegrated with the selection gene replication of this gene give rise to replication of the gene encoding the desired

protein. The result is that increased copies of the gene, i.e., an amplified gene, encoding the desired heterologous protein express more of the desired heterologous protein.

Preferred host cells for expressing the CD antigen variants of this invention are mammalian cell lines, examples including: monkey kidney CF1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293, Graham, F.L. *et al.*, *J. Gen. Virol.* **16**:59 [1977] and 2938 cells [293 subclones selected for better suspension growth]); baby hamster kidney cells (BHK, ATCC CCL 10); chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* **77**:4216, [1980]); mouse sertoli cells (TM4, Mather, J.P., *Biol. Reprod.* **23**:243-251 [1980]); monkey kidney cells (CF1 ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51 cells); and TRI cells (Mather, J.P., *et al.*, *Annals N.Y. Acad. Sci.* **383**:44-68 [1982]).

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. One suitable for transformation of the host cells is the method of Graham, F. and van der Eb, A., *Virology* **52**:456-457 (1973). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell walls are used as hosts, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F.N. *et al.*, *Proc. Natl. Acad. Sci. USA* **69**:2110 (1972).

Construction of suitable vectors containing the desired coding and control sequences employ standard and manipulative ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and reassembled in the form desired to form the plasmids required. Suitable procedures are well known for the construction described herein. See, for example, (Maniatis, T. *et al.*, *Molecular Cloning*, 133-134 Cold Spring Harbor, [1982]; "Current Protocols in Molecular

Biology," edited by Ausubel et al., [1987], publ. by Greene Publishing Associates & Wiley-Interscience).

5                   Correct plasmid sequences are confirmed by transforming E. coli K12 strains 294 (ATCC 31446) with ligation mixtures, successful transformants selected by ampicillin or tetracycline resistance where appropriate, plasmids from the transformants prepared, and then analyzed by restriction enzyme digestion and/or sequenced by the method of Messing et al., Nucleic Acids Res. 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology 65:499 (1980).

10                  Host cells are transformed with the expression vectors of this invention. Thereafter they are cultured in appropriate culture media, e.g., containing substances for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

15                  The secreted polypeptides (including bispecific antibodies) are recovered and purified from the culture supernatants or lysates of recombinant hosts. Typically, the supernatants are concentrated by ultrafiltration, contacted with a ligand affinity or immunoaffinity matrix so as to adsorb the adheson variant, and eluted from the matrix. Optionally, the bispec or adheson is purified by ion exchange chromatography.

20                  The purified bispec is formulated into conventional pharmacologically acceptable excipients. It is administered to patients having HIV infection at a dosage capable of maintaining a concentration of greater than about 100 ng of soluble bispec/ml plasma. For variants having different molecular weights, about 2 picomoles of soluble receptor per ml of plasma will be initially evaluated clinically in order to establish a stoichiometric equivalence with native (membrane bound) and soluble receptor. The ordinary initial dosage of a bispec is 100  $\mu$ g/kg of patient weight/day. This initial dosage can be adjusted either upward or downward depending on the effects of the initial dosage.

25                  30                  A composition of the invention for use in vivo generally will contain a pharmaceutically acceptable carrier. By this is intended either solid or liquid material, which may inorganic or organic and of synthetic or natural origin,

with which the active component of the composition is mixed or formulated to facilitate administration to a subject. Inert materials in pharmaceutical compositions are also called excipients. Any other materials customarily employed in formulating pharmaceutical are suitable. Solid carriers include natural and synthetic cloisonne silicates, for example natural silicates such as diatomaceous earths; magnesium silicates, for example, talcs; magnesium aluminum silicates, for example attapulgites and vermiculites; aluminum silicates, for example kaolinates, montmorillonites, and micas; calcium carbonates; calcium sulfate; synthetic hydrated silicone oxides and synthetic calcium or aluminum silicates; elements such as carbon or sulfur; natural and synthetic resin such as polyvinyl alcohol; and waxes such as parafan and beeswax. Examples of suitable liquid carriers include water and aqueous solutions containing oxygenated organic compounds such as ethanol.

The therapeutic bispec can be employed by itself or with other therapies and agents for the treatment of AIDS, including AZT, neutralizing antibodies, immunocytotoxins, and vaccines.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available, publicly available on a restricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a

larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a 5 polyacrylamide gel to isolate the desired fragment.

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, 10 removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally (Lawn, R. *et al.*, Nucleic Acids Res. 9:6103-6114 [1981], and Goeddel, D. *et al.*, Nucleic Acids Res. 8:4057 [1980]).

"Dephosphorylation" refers to the removal of the terminal 5' 15 phosphates by treatment with bacterial alkaline phosphatase (BAP). This procedure prevents the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Procedures and reagents for dephosphorylation and other recombinant manipulations are conventional. 20 Reactions using BAP are carried out in 50mM Tris at 68°C to suppress the activity of any exonuclease which may be present in the enzyme preparations. Reactions were run for 1 hours. Following the reaction the DNA fragment is gel purified.

"Ligation" refers to the process of forming phosphodiester bonds 25 between two double stranded nucleic acid fragments (maniatis, T. *et al.*, *Id.* at 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

"Filling" or "blunting" refers to the procedures by which the single 30 stranded end in the cohesive terminus of a restriction enzyme-cleaved nucleic acid is converted to a double strand. This eliminates the cohesive terminus and forms a blunt end. This process is a versatile tool for converting a restriction cut end

that may be cohesive with the ends created by only one or a few other restriction enzymes into a terminus compatible with any blunt-cutting restriction endonuclease or other filled cohesive terminus. Typically, blunting is accomplished by incubating 2-15  $\mu$ g of the target DNA in 10mM MgCl<sub>2</sub>, 1mM dithiothreitol, 5 50mM NaCl, 10mM Tris (pH 7.5) buffer at about 37°C in the presence of 8 units of the Klenow fragment of DNA polymerase I and 250  $\mu$ M of each of the four deoxynucleoside triphosphates. The incubation generally is terminated after 30 min. phenol and chloroform extraction and ethanol precipitation.

10 The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention. Examples 1-4 are prior examples published in PCT/US88/03414 which show preparation of antibody/CD4 fusions that can be used to prepare bispecs of the invention, such as those described in Example 5.

15

Example 1Construction of Vectors for the Expression of Native CD4 and Secreted Derivatives20 Section 1

The plasmid used for recombinant synthesis of human CD4 was pSVeCD4DHFR. The plasmid was constructed as follows:

25  $\lambda$ CD4PI containing most of the coding sequence of human CD4 (obtained from a human placental cDNA library using oligonucleotide probes based on the published CD4 sequence [Maddon et al. 1985]) was digested with EcoRI to produce cDNA insert. This fragment was recovered by polyacrylamide gel electrophoresis (Fragment 1).

30 pUC18 was digested with EcoRI and the single fragment recovered by polyacrylamide gel electrophoresis (Fragment 2). Fragment 1 was ligated to fragment 2 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by

restriction analysis for the presence of the correct DNA fragments. This plasmid is referred to as pUCD4.

5 pSVeE'DHFR (Muesing et al., Cell 48:691-701 [1987]) was digested with KpnI and BamHI and blunted with E. coli DNA polymerase I (Klenow fragment) and the four dNTPs. Fragment 3 containing the pML-Amp<sup>r</sup> region, SV40 early promoter, the HIV LTR, and the mouse DHFR gene was recovered by gel electrophoresis, ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the BamHI restriction site and the absence of the KpnI restriction site. This plasmid is referred to as pSVeΔBKDHFR and allows EcoRI-BamHI fragments to be inserted after the SV40 early promoter and transcribed under its control, following transfection into an appropriate cell line.

10 15 Synthetic oligonucleotides (adaptors 1-8, below) were made to extend from 76 bp 5' of the initiation codon of CD4 translation to the RaaI restriction site at 121 bp 3' of the initiator, with the sequence AATT at the 5' end of the sense strand to generate an end which could ligate to an EcoRI restriction fragment. These oligonucleotides were ligated and the 204 bp fragment containing the entire sequence recovered by gel electrophoresis (Fragment 4).

20 25 30 CD4 adaptor 1: AATTCAAGCCCAGAGCCCTGCCATTTCTGTGGGCTC  
AGGTCCCT  
CD4 adaptor 2: PACTGCTCAGCCCCCTCCCTCCCTCGGCAAGGCCACA  
ATGAACCAGGGAGTC  
CD4 adaptor 3: pCCTTTAGGCACITGCTTCTGGTGCTGCAACTGGCG  
CTCCTCCCAGC  
CD4 adaptor 4: pAGCCACTCAGGGAAACAAAGTGGTGCTGGCAAAA  
AGGGGGATACAGTGGAAAGTGAACCTGT  
CD4 adaptor 5: pACAGGTCACTTCCACTGTATCCCCTTTTGCCAG  
CACCACTTTGTTCC

CD4 adaptor 6: pCTGAGTGGCTGGAGGAGCGCCAGTTGCAGCA  
CCAGAACAGT

CD4 adaptor 7: pGCCTAAAAGGGACTCCCCGGTCATTGTGGCCTTG  
CGAGGGAGGAAGGG

5 CD4 adaptor 8: GCTGAGCAGTAGGGACCTGAGCCCACAGAAATGGCAGG-  
GCTCTGGGCTTG

pUCCD4 was digested with RsaI and SstI and the 401 bp fragment containing part of the CD4 coding sequence recovered by gel electrophoresis (Fragment 5). pUC18 was digested with EcoRI and SstI and the fragment comprising the bulk of the plasmid recovered by gel electrophoresis (Fragment 6).  
10 Fragments 4 and 5 were ligated to Fragment 6 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence  
15 of the correct fragment. The sequence of the inserted synthetic DNA was checked by excising the 605 bp EcoRI-SstI fragments from several transformants and ligating them to M13mp19 which had been digested with the same enzymes. After transformation into E. coli strain JM101, single-stranded DNA was prepared and sequenced. One plasmid which contained the correct sequence was selected, and  
20 is referred to as pCD4int.

25 pCD4int was digested with EcoRI and SstI and fragment 7 containing the 5' end of the CD4 coding region was recovered by gel electrophoresis. pUCCD4 was digested with SstI and BamHI and the 1139 bp fragment containing the remainder of the CD4 coding region (fragment 8) recovered by gel electrophoresis.

30 pSV $\Delta$ BKDHF was digested with EcoRI and BamHI and fragment 9 comprising the bulk of the plasmid was isolated. Fragments 7, 8, and 9 were ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and the resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is

referred to as pSVeCD4DHFR, and was used to direct synthesis of recombinant intact CD4.

Example 2

5

Fusions of the V region of the CD4 gene, which is homologous to the variable region of immunoglobulin genes (ref. Madden *et al.* 1985), to the constant (C) region of human immunoglobulin  $\kappa$  and  $\gamma 2$  chains are constructed as follows:

10

Synthetic DNA is made to code for the C region of human  $\kappa$  chain (residues 109-214) based on the sequence published by Morin *et al.*, Proc. Natl. Acad. Sci. 82:7025-7029, with the addition at the 5' end of the coding strand of the sequence GGGG, which allows this fragment to be ligated to the BspMI site at the end of the putative V-like region of CD4. At the 3' end of the coding region, a translational stop codon is added as well as a sequence which allows this end to be ligated to BamHI restriction fragments. The synthetic DNA is made in 8 fragments, 4 for each strand, 70-90 bases long. These are then allowed to anneal and ligated prior to isolation on a polyacrylamide gel (fragment 33).

15

pRKCD4 is digested with EcoRI and BspMI and the 478bp fragment containing the region coding for the putative V-like domain of CD4 is recovered (fragment 34). Fragments 33 and 34 are ligated together with fragment 16 (from the expression vector pRK5). The ligation mixture is transformed into E. coli strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA is prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4C $\kappa$ .

20

A plasmid encoding a fusion of the CD4 V-like domain to the human immunoglobulin C $\gamma 2$  region is constructed in a similar fashion, and is referred to as pRKCD4C $\gamma 2$ . Both of these plasmids are transfected into 293 cells, myeloma cells or other competent cells in order to obtain cell lines expressing variant CD4 molecules as described above.

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Example 3

Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin  $\gamma 1$ . These plasmids are referred to as pRKCD4<sub>2 $\gamma 1$</sub> , pRKCD4<sub>e4 $\gamma 1$</sub> , pRKCD4<sub>2 $\gamma 1$</sub> , pRKCD4<sub>1</sub>, and pRKCD4<sub>e1 $\gamma 1$</sub> .

5

Plasmid pRKCD4<sub>4 $\gamma 1$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for serine residue 366 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat *et al.*).

10

Plasmid pRKCD4<sub>e4 $\gamma 1$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for lysine residue 360 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat *et al.*).

15

Plasmid pRKCD4<sub>2 $\gamma 1$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for glutamine residue 180 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat *et al.*).

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Plasmid pRKCD4<sub>e2 $\gamma 1$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 117 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat *et al.*).

25

Plasmid pRKCD4<sub>1 $\gamma 1$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for aspartic acid residue 105 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat *et al.*).

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Plasmid pRKCD4<sub>e1 $\gamma 1$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 100 of the

mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat *et al.*).

5 Construction of these plasmids required the prior construction of plasmid pRKCD4TP/ $\gamma 1$ . It was constructed as follows:

A cDNA clone coding for human immunoglobulin  $\gamma 1$  was obtained from a human spleen cDNA library (Clontech Laboratories, Inc.) using oligonucleotides based on the published sequence (Ellison *et al.*, Nucl. Acids Res. 10:4071-4079 [1982]), and an EcoRI-EagI fragment (the EcoRI site was contributed by a linker; see Figure 4a and b of PCT/US88/03414) containing part of the variable and all of the constant region was obtained. This fragment was blunted with Klenow fragment, and recovered by gel electrophoresis (Fragment al).

10 15 Plasmid pRKCD4TP-kk, encoding a substitutional variant of soluble CD4 (residues 1-368) containing a lysine residue instead of asparagine at position 1 of the mature polypeptide, was constructed from plasmid pRKCD4TP by site-directed mutagenesis. A synthetic oligonucleotide was made as a primer for a mutagenesis reaction to obtain the desired coding sequence. This was synthesized as a 51-mer which contained two silent mutations from the natural sequence in addition to the substitution mutation, and 21 bases on each side of the mutated codons:

20 25 30 5' - CCC TTT TTT GCC CAG GAC CAC CTT CTT GCC CTG-  
AGT GGC TGC TGG GAG GAG -3'

Plasmid pRKCD4TP was transformed into E. coli strain SR101 and the transformed colonies plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of m13K07 helper bacteriophage to yield secreted, encapsidated single-stranded templates of pRKCD4TP. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reaction was transformed E. coli SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony

hybridization (ref. Grunstein-Hogness) for the presence of the appropriate sequence, using the following 16 mer as the probe.

5' - C CAC CTT CTT GCC CTG -3'

5 The hybridization conditions chosen were sufficiently stringent that the probe only detects the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into E. coli strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13K07 bacteriophage. Templates were prepared as above and screened by sequencing.

10 Plasmid pRKCD4TP-kk was digested with XbaI and treated with Klenow Enzyme, and Fragment a2, containing the linearized plasmid was recovered by gel electrophoresis, and ligated with fragment a1. The ligation mixture was transformed into E. coli strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from the transformants and checked by restriction analysis for the presence of the correct fragment in the correct orientation (i.e., the immunoglobulin coding region in the same orientation as the CD4 coding region, and at the 3' end of the CD4 coding region). This plasmid is referred to as pRKCD4TP/γ1.

15 20 Synthetic oligonucleotides were made as primers for deletional mutagenesis reactions to fuse the appropriate coding sequence of IgG1 and CD4 as described above. These were synthesized as 48-mers comprising 24 nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired immunoglobulin moiety). Plasmid pRKCD4TP/γ1 was transformed into E. coli strain SR101 and the transformed cultures plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of m13K07 helper bacteriophage to yield secreted, encapsidated single-stranded templates of pRKCD4TP/γ1. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reactions were transformed E. coli SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (ref. Grunstein-

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Hogness) for the presence of the appropriate fusion site, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into *E. coli* strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13K07 bacteriophage. Templates were prepared as above and screened by sequencing.

The plasmids were transfected into 293 cells using standard procedures and assayed for expression and production as described above.

		<u>Expressed</u>	<u>Secreted</u>
	pRKCD4 <sub>1γ1</sub>	+	-
15	pRKCD4 <sub>e2γ1</sub>	+	+
	pRKCD4 <sub>2γ1</sub>	+	+
	pRKCD4 <sub>e4γ1</sub>	+	+
	pRKCD4 <sub>4γ1</sub>	+	+

20 Plasmids also were constructed to direct the expression of fusion proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the truncated portion of the constant region of human immunoglobulin  $\gamma$ 1, comprising only the hinge region and constant domains  $CH_2$  and  $CH_3$ .

25 Synthetic oligonucleotides were made as primers for mutagenesis reactions to delete the immunoglobulin sequence from Ser114 to Cys215 inclusive (Kabat *et al.*). These were synthesized as 48-mers comprising 24 nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired CD4 moiety, and the  $NH_2$ -terminal 8 residues of the desired immunoglobulin moiety). Plasmids pRKCD4<sub>4γ1</sub>, pRKCD4<sub>2γ</sub> and the transformed culture plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of m13K07 helper bacteriophage to yield

secreted, encapsidated single-stranded templates of these plasmids. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reactions were transformed *E. coli* SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (Grunstein-Hogness) for the presence of the appropriate fusion sites, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into *E. coli* strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13K07 bacteriophage. Templates were prepared as above and screened by sequencing.

The plasmid derived from plasmid pRKCD4<sub>4γ1</sub> is referred to as pRKCD4<sub>4Fcl</sub>, that derived from plasmid pRKCD4<sub>2γ1</sub> is referred to as pRKCD4<sub>2Fcl</sub> and that derived from plasmid pRKCD4<sub>1γ1</sub> is referred to as pRKCD4<sub>1Fcl</sub>.

pRKCD4<sub>2Fcl</sub>, pRKCD4<sub>1Fcl</sub> and pRKCD4<sub>4Fcl</sub> are cultured in the same fashion as described above and CH1-deleted CD4 immunoadhesons recovered as described elsewhere herein.

#### Light Chain Fusions

Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin κ. These plasmids are referred to as pRKCD4<sub>4κ</sub> and pRKCD4<sub>e4κ</sub>.

Plasmid pRKCD4<sub>4κ</sub> contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for serine residue 366 of the mature CD4 polypeptide, immediately followed by the sequence for the constant region of human immunoglobulin κ, starting at the codon for threonine residue 109 of the mature human immunoglobulin κ. (Kabat *et al.*)

Plasmid pRKCD4<sub>e4 $\kappa$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for lysine residue 360 of the mature CD4 polypeptide, immediately followed by the sequence for the constant region of human immunoglobulin  $\kappa$ , starting at the codon for threonine residue 109 of the mature human immunoglobulin  $\kappa$ . (Kabat et al.)

These plasmids were constructed in a manner analogous to plasmids pRKCD4<sub>4γ1</sub> and pRKCD4<sub>e4γ1</sub> described above, with the following exception:

The human immunoglobulin  $\kappa$  coding sequence (Figure 5 of PCT/US88/03414) was obtained from a human spleen cDNA library (Clontech Laboratories, Inc.) using oligonucleotides based on the published sequence (Hieter, P.A. et al., Cell 22:197-207 [1980]), and an EcoRI-BspMI fragment containing part of the variable region and the entire constant region was obtained (see Figure 5 of PCT/US88/03414). This fragment was blunted with Klenow fragment and the four dNTPs. This fragment was used instead of fragment a1, and was used to construct plasmid pRKCD4TP/br.

### Expression in CHO Cells

Plasmids were or are constructed to direct the expression of the immunoadhesons described above in CHO cells. These are referred to as pSVeCD4<sub>4 $\gamma$</sub> SVDHFR, pSVeCD4<sub>2 $\gamma$ 1</sub>SVDHFR, pSVeCD4<sub>1 $\gamma$</sub> SVDHFR, pSVeCD4<sub>34 $\gamma$ 1</sub>SVDHFR, pSVeCD4<sub>e2 $\gamma$ 1</sub>SVDHFR, pSV3CD4<sub>e1 $\gamma$ 1</sub>SVDHFR, pSVeCD4<sub>4Fc1</sub>SVDHFR, pSVeCD4<sub>2Fc1</sub>SVDHFR, pSVeCD4<sub>1Fc1</sub>SFCHFR, pSVeCD4<sub>4 $\kappa$</sub> SVDHFR and pSV3CD4<sub>2 $\kappa$</sub> SVDHFR.

25. Fragment 31 was prepared as described above. Fragment 32a was prepared by digesting plasmid pE348HBV.E400 D22 with BamHI, blunting with Klenow fragment and the four dNTPs, then digesting with PyuI and isolating the large fragment containing the balance of the  $\beta$ -lactamase gene and the SV40 early promoter and the DHFR gene. Plasmids pRKCD4<sub>4 $\gamma$ 1</sub>, pRKCD4<sub>2 $\gamma$ 1</sub>,  
 30 pRKCD4<sub>1 $\gamma$ 1</sub>, pRKCD4<sub>e4 $\gamma$ 1</sub>, pRKCD4<sub>e2 $\gamma$ 1</sub>, pRKCD4<sub>e1 $\gamma$ 1</sub>, pRKCD4<sub>4Fcl</sub>, pRKCD4<sub>2Fcl</sub>, pRKCD4<sub>4 $\kappa$</sub>  and pRKCD4<sub>2 $\kappa$</sub>  were separately digested with HindIII, blunted with Klenow fragment and the four dNTPs, then digested with EcoRI and the fragments encoding the CD4-Ig fusion protein were isolated. The resulting

DNA fragments were ligated together with fragments 31 and 32a and transformed into *E. coli* strain 294. Colonies were selected and checked for the presence of the correct plasmid as above, then transferred into CHO cells and amplified by methotrexate selection using conventional procedures.

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### Example 4

## Culture, Purification and formulation of CD4 variants

10 Plasmids encoding soluble CD4 immunoadhesons were calcium phosphate transfected into CHO-DP7 (a proinsulin-transformed autocrine host cell derived from CHO; U.S.S.N. 97,472) and the transformants grown in selective medium (1:1 HAM F12/DMEM GHT containing 1-10% diafiltered or dialyzed bovine serum). Other suitable host cells are CHO cells or 293S human embryonic kidney cells. The transformants were amplified by methotrexate. The plasmid that was integrated in the genome of the transfectoma was generated by cutting out an EcoRI - SfiI fragment from the CD4 $\gamma$ 1 plasmid, filling the ends, and blunt end-ligating the fragment into the XbaI site of the pcDEB vector, which contains the hygromycin resistance gene.

20 The general procedure for purifying a variety of CD4 proteins was  
as follows. Specific parameter values (e.g., pH) in this paragraph are for a  
soluble CD4 variant consisting essentially of the first four domains (expressed in  
soluble form) known as CD4TP. Culture fluid from CD4 transformants was  
concentrated and diafiltered to lower the ionic strength. The concentrate was  
25 passed through a large volume of Q-Sepharose anion exchange resin (previously  
equilibrated with 25 mM NaCl, pH 8.5) in order to adsorb contaminants from the  
culture fluid. The unadsorbed culture fluid from the anion exchange resin step  
was then passed through a cation exchange resin (previously equilibrated with 25  
mM NaCl at pH 8.5) whereby the CD4 variant was adsorbed to the resin. The  
30 protein was eluted with a NaCl gradient at pH 8.5. Ammonium sulfate was  
added to the eluate to a concentration of 1.7M and the solution passed through a  
column of hydrophobic interaction chromatography resin (phenyl or butyl

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Sepharose). The protein was eluted from the hydrophobic interaction column with a gradient of ammonium sulfate. The eluate was concentrated and buffer exchanged on a G-25 column using phosphate buffered saline containing .02% (w/v) Tween 20 or Tween 80. Other polymeric nonionic surfactants are suitably used with the CD4 formulations, including Pluronic block copolymers or polyethylene glycol.

10

It is also possible to employ immunoaffinity purification of soluble CD4 variants wherein the CD4 is adsorbed onto an immobilized antibody against CD4. This method suffers from the disadvantage that elution of the soluble CD4 under acidic conditions leads to protein aggregation that is only thoroughly ameliorated at relatively higher levels of surfactant. The foregoing procedure permits the use of much lower quantities of surfactant, about from 0.01 to 0.10% (w/v) surfactant.

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The procedure followed for the purification of CD4 fusions with immunoglobulin heavy chain was to concentrate recombinant supernatants by ultrafiltration and thereafter adsorb the fusion onto resin-immobilized Staphylococcal protein A. The fusion was eluted with 0.1M citrate buffer pH 3 with no salt or detergent. This preparation is buffered into Tris buffer at pH 7.5. The immunoglobulin fusions with CD4 V1-V4 optionally are further purified by the procedure described above for unfused CD4 variants (i.e., the general procedure of this Example). CD4 immunoglobulin fusions with CD4 V1-V2 also may be purified by the procedure above, except that it is not expected that the isoelectric point of this class of molecules will be as alkaline as that of species containing all four V regions of CD4.

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#### Example 5

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We cloned the gene encoding the fusion polypeptide CD4 $\gamma$ 1 (CD4 V1-V4 fused to  $\gamma$ 1) described above into a vector that allows expression in mouse cells and transfected it into hybridoma M-T301, which secretes a  $\gamma$ 1,  $\kappa$  anti-human CD3 antibody. This antibody can activate T cells when added in polymerized form. This particular hybridoma and anti-human CD3 antibody were selected for convenience only. The same transfection process can be carried out with other

hybridomas that produce anti-CD3 antibodies in order to produce bispecific antibodies of the invention.

The bispecific antibody produced in this example is shown schematically in Figure 2. Figure 3a shows an autoradiograph of biosynthetically labeled intracellular proteins obtained from the hybridoma of this invention precipitated with antibody to mouse  $\gamma 1$  chain (lanes 2 and 3). A 92 kd polypeptide representing CD4 $\gamma 1$  is precipitated from the transfected (lane 3), but not from the untransfected cell line (lane 2). Some of the CD4 $\gamma 1$  is secreted (Figure 3b) in association with the  $\kappa$  chain of M-T301, this dimer being linked to the heavy(H) X light(L) chain pair of M-T301. In addition, there are other combinations of the three polypeptide chains present in the transfectoma; this can be deduced from the molecular weights of the unreduced molecules (Figure 3c, lane 4). Furthermore, we have rerun lane 4 of Figure 3c under reducing conditions, confirming that some of the molecules contain CD4 $\gamma 1$ , H and L chain (Figure 3d).

From the supernatant of the transfectoma, we then purified bispecs by binding them to and later eluting them from an affinity column consisting of monoclonal antibody to CD4, which separated them from bivalent anti-CD3 antibodies, which passed through the column. Electrophoresis of this material on a polyacrylamide gel revealed equal proportions of two kinds of molecules: a bivalent monospecific tetramer consisting of CD4 $\gamma 1$  and  $\kappa$ , and the desired bispec with the composition  $\kappa$ , CD4 $\gamma 1$ ,  $\gamma$ ,  $\kappa$  (Figure 3e). Thus the CD4 $\gamma 1$  chain is linked to  $\kappa$  and pairs with the H x L chain half of IgG1 molecule of M-T301 (Figure 3f). The rather large CD4 $\gamma 1$  is not sterically hindered from being linked to the  $\gamma 1$  chain, probably because of the flexibility of the hinge region, which allows the amino ends of the polypeptides to move apart. The  $\kappa$  chain is also not hindered from forming a disulfide bridge with the CD4 $\gamma 1$  chain.

The ability of the bispecs to mediate killing of HIV-infected cells by nonspecific cytotoxic T cells was tested with a chromium release assay that we developed. As target cells, we used CD4-expressing HeLa cells, which we had infected with HIV. The percentage of infected cells was assessed by immunofluorescence using a monoclonal antibody to viral p24 in the cytoplasm

and the monospecific CD4g1,  $\kappa$  antibody for testing expression of membrane gp120. From the bulk culture we subcloned lines in which greater than 90% of the cells stably expressed these proteins. As effector cells we used clones of the human cytotoxic T cell line MX66, which is specific for influenza virus matrix protein when presented on human cells expressing the histocompatibility antigen A2. At an effector:target ratio of 1:1 the MX66 cells did not show significant killing of the CD3-positive HUT 78 cell line; however, when M-T301 antibody, which is specific to CD3, was added, good killing was seen. (Table 3). This confirms that M-T301 antibody is able to mediate the killing of non-cognate target cells, by activating the MX66 cells and linking them to these targets.

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Thus, this is a suitable system for testing the bispecs.

5           In the experimental work that provided the data set out in Table 3, target cells were incubated with antibodies at one or more concentrations (or with no antibody), washed, and cells of the cytotoxic T cell line MX66 (effector cells) obtained from H. Spits, DNAX, Palo Alto) were added at a ratio of 0.5:1 or 1:1, or as a control, medium with no effector cells was added (no E). Targets were one of the following: HeLa cells expressing CD4 and infected with HIV-LAV I (HeLa LAV I); or not infected (HeLa); or allogeneic cell line HUT 78. Antibodies were one of the following: anti-CD3 monoclonal antibody M-T301; 10 bispecific antibodies (bispecs) with one arm specific for CD3 (derived from M-T301) and the other arm specific for gp120 (derived from CD4g1); a 1:1 mixture of M-T301 and CD4g1 (antibody mixture). The values in the body of the table are the percentages of cells killed (resulting  $^{51}\text{Cr}$  release) as calculated from the formula: %lysis =  $(\text{cpm experimental release} - \text{cpm spontaneous release}) : (\text{cpm maximal release} - \text{cpm spontaneous release}) \times 100$ . Spontaneous release was 15 always less than 15% of maximal release. Maximum release was determined after lysis with 1N HCl.

20           In Experiment II the background killing, i.e., chromium release in the presence of effector cells but in the absence of antibodies, is higher than in Experiment I. This commonly observed non-specific killing by T cell clones is thought to depend on culture conditions. Furthermore, in Exp. II the mixture of monospecific antibodies also resulted in some killing of the infected, but not the uninfected, cells. In Exp. II the M-T301 antibody was purified from transfectoma supernatant and presumably contained small amounts of bispecs. In 25 Exp. I, the M-T301 antibody was purified from M-T301 hybridoma supernatant, and thus must have been free of such contamination.

30           In summary, Table 3 shows that bispecs are effective in killing HIV-infected cells *in vitro*. In Experiment I HeLa.T4 cells, whether HIV-infected or not, were not killed by effector cells alone. However, when we added our bispecs at a concentration of 1.6  $\mu\text{g}/\text{ml}$ , 26.8% of infected cells were killed at an effector:target ratio of 0.5:1, while the uninfected cells were not killed. At an effector:target ratio of 1:1, 40% of the infected, but only 2% of

uninfected cells were killed. A mixture of equal parts of bivalent CD4 $\gamma$ 1, $\kappa$  tetramers and M-T301 antibodies did not cause killing (below 0.7%). The effect of the bispecs diminished at a lower concentration and was absent at 16 ng/ml. However, in Experiment II this concentration was still effective (in another experiment not shown, 1.6 ng/ml showed some effect). As mentioned above, the bispec preparation contained an equal amount of monospecific bivalent CD4 $\gamma$ 1, $\kappa$  tetramers, which do not help the killing, but which, due to their superior avidity (two binding sites vs. one), may rather prevent bispecs from binding to the target cells. Further purification of the bispecs should yield preparations that are somewhat more efficient. To alleviate the necessity for a tedious purification, mutations in the  $\gamma$  chains that allow only heterologous pairing, i.e. V<sub>H</sub> $\gamma$ 1 with CD4 $\gamma$ 1, can be selected.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

Table 3 (page 1)

Antibody	conc. μg/ml	% specific $^{51}\text{Cr}$ release from target cells							
		IIUT 78				IIcl-a-LAV 1			
		Effector:Target		Effector:Target		Effector:Target		Effector:Target	
		0.5:1	1:1	0.5:1	1:1	no E	0.5:1	1:1	no E
no antibody	..	0	0.4±0.2	0	0.7±1.2	..	1.6±1.7	1.7±0.8	..
M-T301	~2	34.2±2.3	48.3±1.9	..	..	..	..	..	..
bispec	1.6	..	26.8±3.0	40.3±3.3	0	0.3±0.6	2.2±0.4	0	..
0.16	..	..	9.8±1.7	14.6±2.1	0.9±1.2	0.8±0.8	0.5±0.6	0	..
0.016	..	..	0.9±1.6	3.2±1.5	0	0.4±0.1	1.7±1.8	0	..
0.0016	..	..	2.3±0.9	0.9±1.2	0	0	0	0	..
0.00016	..	..	0.9±0.6	2.0±2.2	0	0.2±0.3	0.9±1.4	0	..
antibody mixture	1.6	..	..	0	0	0.2±0.9	0.7±0.8	0	..
	0.16	..	..	0	0.2±1.8	0	0	0.3±0.9	0

Table 3 (page 2)

Antibody	conc. μg/ml	% specific $^{51}\text{Cr}$ release from target cells							
		HUT 78				HeLa-LAV 1			
		Effector:Target		Effector:Target		Effector:Target		Effector:Target	
		0.5:1	1:1	0.5:1	1:1	no E	0.5:1	1:1	no E
no antibody	--	0.3±0.8	1.5±0.5	3.5±1.6	8.2±1.9	--	0	0	--
M-T301	~2	53.3±1.2	64.6±4.0	--	--	--	--	--	--
bispec	1.6	--	--	49.0±5.4	67.2±3.2	0.8±0.7	4.3±1.9	6.0±1.5	0
	0.16	--	--	31.6±3.5	44.6±2.8	1.0±0.8	0	1.1±2.1	0
	0.016	--	--	10.7±0.4	16.0±2.5	0.4±1.3	0	0.8±2.0	0
	0.0016	--	--	3.1±2.0	5.7±0.2	0	0	2.7±1.3	0
	0.00016	--	--	1.9±1.7	7.0±1.0	0	0	0	0
antibody mixtue	1.6	--	--	11.1±0.5	17.5±1.2	0	0	0	0
	0.16	--	--	6.6±0.5	11.3±1.1	0.1±0.7	0	0	0

## WHAT IS CLAIMED IS

5 1. A method for directing a cytotoxic T cell to an HIV-infected cell, which comprises:  
contacting said infected cell with a bispecific proteinaceous molecule comprising two binding domains, wherein said first binding domain comprises a CD4 region that binds to HIV gp120 and said second binding domain comprises an anti-CD3 binding region, wherein said molecule is other than a molecule consisting essentially of a CD4 variable region domain or domains that bind to both HIV gp120 and class II MHC antigen fused to a complete antibody constant region as one half of a bispecific antibody in which the second half of said bispecific antibody is a complete light/heavy antibody chain pair specific for CD3.

10 2. The method of Claim 1, wherein said bispecific molecule is a bispecific antibody with a deletion of a binding region specific for Fc receptor or a deletion of a CD4 binding region for class II MHC antigen.

15 3. The method of Claim 2, wherein said first binding domain comprises a CD4 domain or domains that is fused to an antibody constant region to form a chimeric antibody chain.

20 4. The method of Claim 3, wherein said second binding domain comprises a variable region domain of an anti-CD3 antibody chain.

25 5. The method of Claim 1, wherein said first binding domain and said second binding domain are different regions of a single polypeptide chain.

30 6. The method of Claim 5, wherein said polypeptide chain comprises an N-terminus and a C-terminus and each of said first and second binding domains is located at one or the other of said termini.

7. The method of Claim 6, wherein said first binding domain is located at said N-terminus and said second binding domain is located at said C-terminus.
- 5 8. The method of Claim 7, wherein said first binding domain contains  $V_1V_2$  domains of CD4.
9. The method of Claim 7, wherein said first binding domain contains  $V_1V_2V_3V_4$  domains of CD4.
- 10 10. The method of Claim 1, wherein said bispecific molecule is present at a concentration of from at least 16 ng/ml in contact with said infected cell.
- 15 11. The method of Claim 1, wherein said cytotoxic T cell is a member of a collection of cytotoxic T cells and said cytotoxic T cells are present at a ratio of from 0.1:1 to 10:1, relative to infected cells, while in contact with said bispecific molecule and HIV-infected cells.
12. The method of Claim 1, wherein said infected cells are located *in vivo*.
- 20 13. A pharmaceutical composition for directing a cytotoxic T cell to an HIV-infected cell *in vivo*, which comprises:  
a bispecific proteinaceous molecule comprising two binding domains, wherein said first binding domain comprises a CD4 region that binds to HIV gp120 and said second binding domain comprises an anti-CD3 binding region and  
25 a pharmaceutically acceptable carrier.
14. The composition of Claim 13, wherein said bispecific molecule is a bispecific antibody.
- 30 15. The composition of Claim 13, wherein said first binding domain consists essentially of a CD4 variable region domain or domains and said second binding

domain consists essentially of an anti-CD3 antibody variable region domain or domains.

5 16. The composition of Claim 13, wherein said first binding domain comprises a CD4 variable region domain or domains that is fused to an antibody constant region to form a chimeric antibody chain.

10

17. The composition of Claim 16, wherein said second binding domain comprises a variable region of an anti-CD3 antibody chain.

15

18. The composition of Claim 13, wherein said first binding domain and said second binding domain are different regions of a single polypeptide chain.

19. The composition of Claim 18, wherein said polypeptide chain comprises an N-terminus and a C-terminus and each of said first and second binding domains is located at one or the other of said termini.

20

20. The composition of Claim 19, wherein said first binding domain is located at said N-terminus and said second binding domain is located at said C-terminus.

21

21. The composition of Claim 13, wherein said first binding domain contains  $V_1V_2$  domains of CD4.

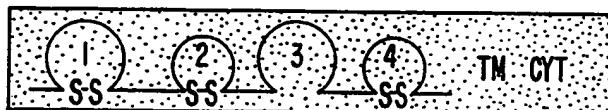
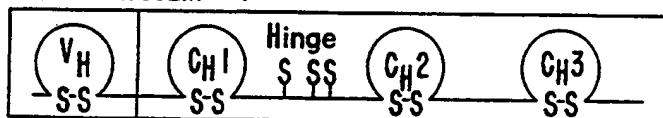
25

22. The composition of Claim 13, wherein said first binding domain contains  $V_1V_2V_3V_4$  domains of CD4.

23. The composition of Claim 13, wherein said composition further comprises a second active component suitable for treating HIV-I infection.

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CD4

IMMUNOGLOBULIN  $\gamma$ 1

SOLUBLE rCD4

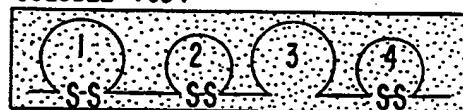
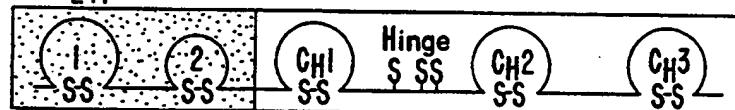
CD4 2 $\gamma$ 1CD4 4 $\gamma$ 1

FIG. I

SUBSTITUTE SHEET

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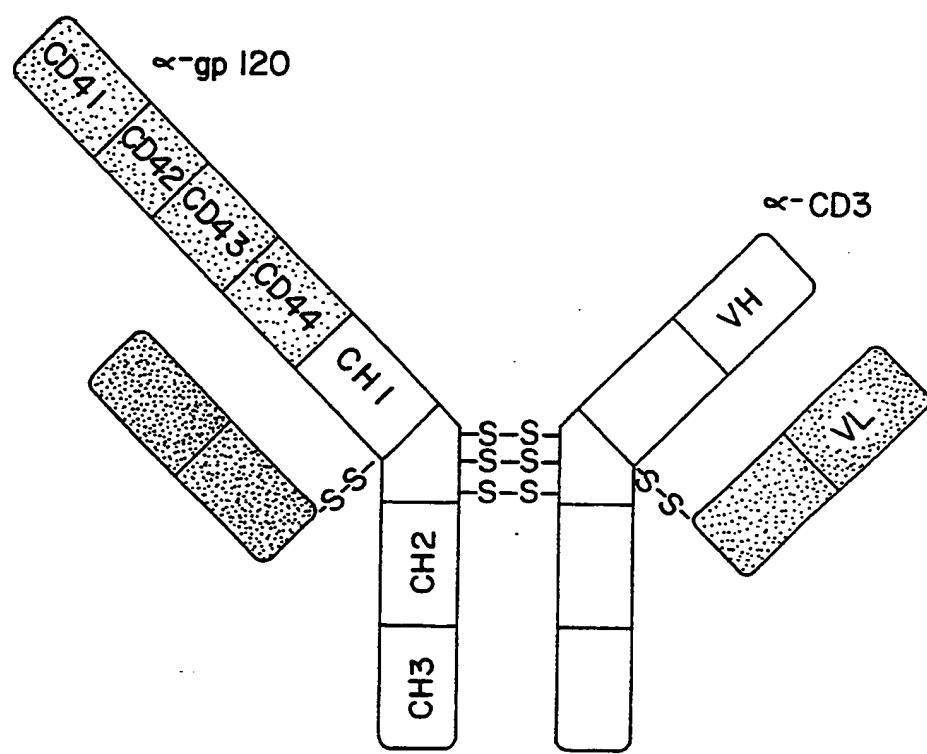


FIG. 2

SUBSTITUTE SHEET

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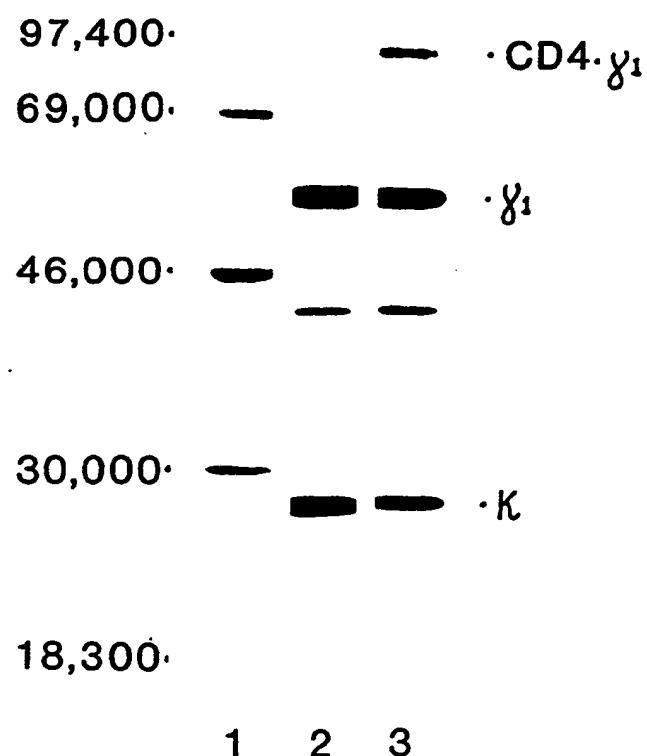


FIG. 3a

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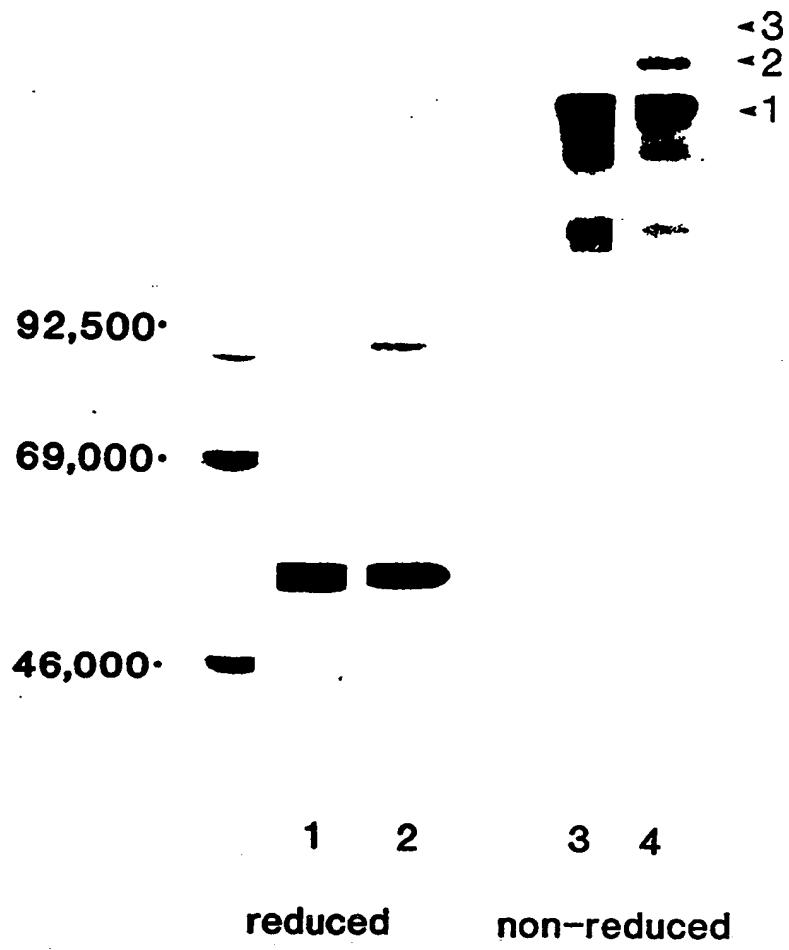


FIG. 3b

FIG. 3c

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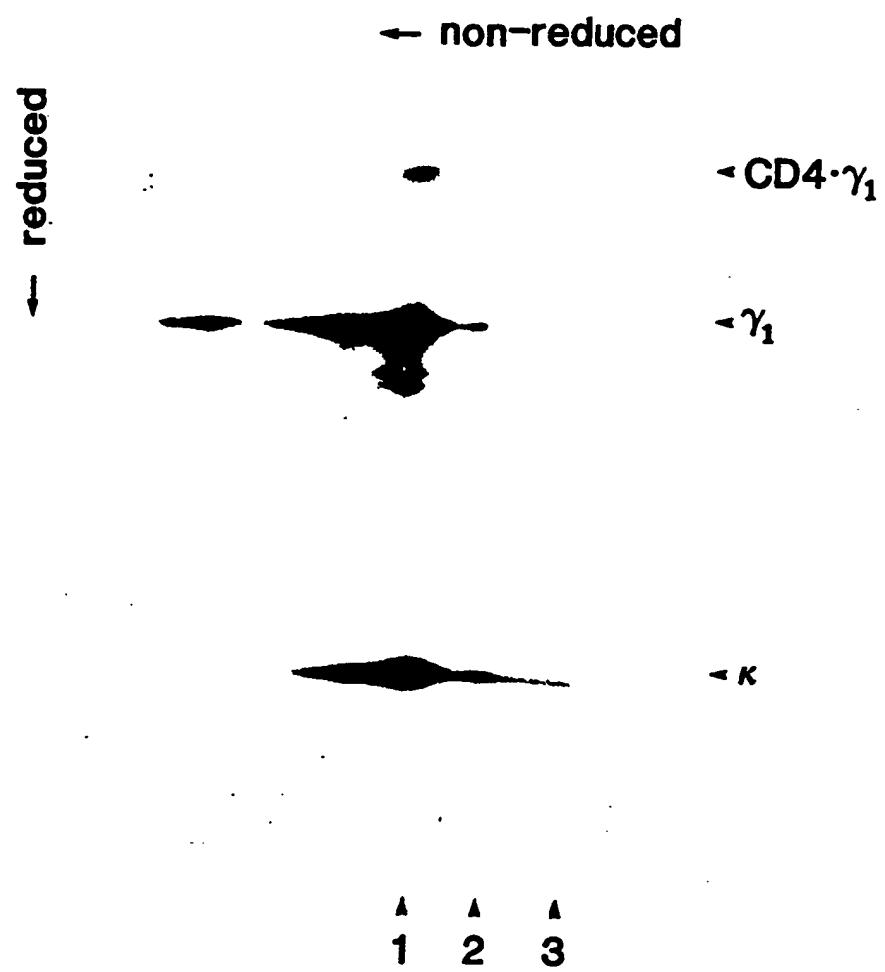


FIG. 3d

SUBSTITUTE SHEET

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■ 2  
■ 1

CD4· $\gamma_1$  ■

$\gamma_1$  ■

reducing      non-reducing

FIG. 3e

SUBSTITUTE SHEET

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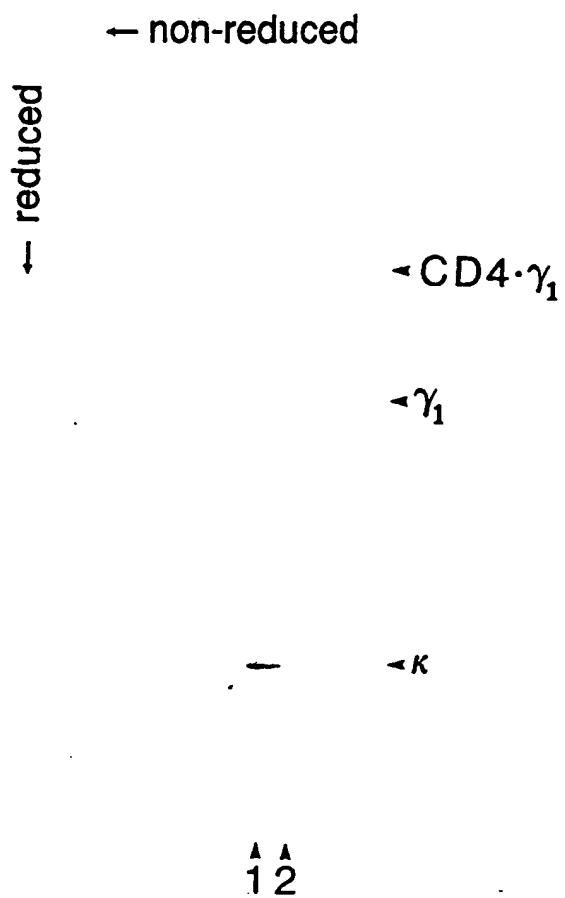


FIG. 3f

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/09550

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61P 37/04  
US CL : 424/85.8

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Int. J. Cancer, Volume 43, Issued 1989, J. van Duk et al., "Induction of tumor-cell Lysis by bi-specific monoclonal antibodies recognizing renal-cell carcinoma and CD3 antigen", pages 344-349, entire document	13-23
Y	Clin. Exp. Immunol., Volume 79, Issued 1990, S. Songsivilai et al., "Bispecific antibody: a tool for diagnosis and treatment of disease", pages 315-321, especially page 318	1-23
Y	Nature, 337, Issued 09 February 1989 (London, GB), D.J. Capon et al., "Designing CD4 immunoadhesins for AIDS therapy", pages 525-530. Entire document	1-23

 Further documents are listed in the continuation of Box C.  See patent family annex.

• Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be part of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is taken alone
"O" document referring to an oral disclosure, use, exhibition or other means	"Z"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P" document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 JANUARY 1993	29 JAN 1993
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